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thanks,
Melissa
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Down-regulation of vascular endothelial growth factor in human colon carcinoma cell lines by antisense transfection decreases endothelial cell proliferation

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Background. *Vascular endothelial growth factor (VEGF) is a potent paracrine angiogenic factor implicated in human colon cancer angiogenesis. This study determined whether regulation of VEGF expression by human colon cancer cell lines affects endothelial cell proliferation in an in vitro model of angiogenesis.*

Methods. *Human colon carcinoma cell lines were screened for VEGF mRNA expression. SW480 cells (low VEGF expresser) were transfected with the VEGF sense (VS) vector, and SW620 cells (high VEGF expresser) were transfected with the VEGF antisense (VAS) vector. Cells were transfected with the vector alone as control. Endothelial cells were grown in conditioned, serum-free medium produced from experimental cells, and proliferation was determined.*

Results. *SW480-VS transfectants exhibited a sixfold increase in VEGF secretion. SW620-VAS transfectants exhibited a 50% reduction in VEGF secretion. Endothelial cells grown in conditioned medium from SW480-VS cells exhibited a 1.5- to 2-fold increase in proliferation. Addition of conditioned medium from SW620-VAS cells resulted in a 25% to 50% decrease in endothelial cell proliferation.*

Conclusions. *VEGF production by human colon cancer cells regulates endothelial cell proliferation. Antisense VEGF transfection can down-regulate VEGF secretion and biologic activity. Therapies to decrease VEGF expression may be a means of inhibiting angiogenesis in primary and metastatic colon cancer. (Surgery 1996;120:871-8.)*

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A LARGE BODY OF WORK by Folkman[1,2] has established that tumor growth is angiogenesis dependent. Angiogenesis is essential for tumor growth to exceed the maximal distance of oxygen transport and nutrient diffusion (less than 1 mm). In addition, angiogenesis must occur for formation and growth of metastases. Tumor angiogenesis is not a passive process. Appropriate factors must be expressed to initiate basement membrane degradation, endothelial cell proliferation and migration, and capillary tubule formation.[3] Increased vascularity may allow not only an increase in tumor growth, but also a greater chance for hematogenous tumor embolization. Thus inhibiting tumor angiogenesis may not only halt tumor growth but may also decrease the metastatic potential of tumors. Identification of specific angiogenic factors in colon cancer may provide a specific target for antineoplastic therapy that may be used alone or in conjunction with other modalities.

Vascular endothelial growth factor (VEGF) (also known as vascular permeability factor) is a 34 to 42 kd protein initially isolated from conditioned media of tumor cell lines.[4,5] This factor was first identified as a potent mediator of vessel permeability.[4] Later studies showed that this factor is also a heparin-binding mitogen specific to endothelial cells.[6] Berse et al.[7] demonstrated expression of VEGF mRNA transcripts in numerous normal and malignant tissues, including colon cancer. In a follow-up publication from this group, strong expression for VEGF was noted in 13 of 17 cases of colon cancer by mRNA in situ hybridization, whereas very little expression was noted in adjacent mucosa.[8]

The gene for VEGF has significant homology to placenta growth factor and platelet-derived growth factor.[9,10] Four different transcripts arise from alternative RNA splicing coding for proteins of 206, 189, 165, and

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121 amino acids in length.[6] VEGF-206 and VEGF-189 are cell-associated proteins, whereas VEGF-165 and VEGF-121 are cell-secreted proteins.[11] VEGF-165 and VEGF-121 are also better mitogens for capillary endothelial cells than VEGF-206 and VEGF-185.[11] Although most tissues predominantly secrete VEGF-165, the most abundant splice variants in primary and metastatic colon cancers are unknown.

The role of VEGF in angiogenesis of human colon cancer has recently been examined in our laboratory. In a study of 62 patients with colon cancer, Takahashi et al.[12] demonstrated that VEGF expression correlates with vascularity, tumor cell proliferation, and metastatic rates. In addition, those patients with high expression of VEGF exhibited an increased incidence of the presence of its receptor (*KDR*) on endothelial cells. The VEGF was found mainly in tumor epithelial cells, and this observation is in agreement with previous studies reported by Brown et al.[8]

If VEGF is the dominant angiogenic factor in human colon cancer, then down-regulation of VEGF should decrease angiogenesis. This study was done to determine whether down-regulation of VEGF by antisense transfection in human colon carcinoma cell lines can decrease endothelial cell proliferation by a paracrine mechanism.

MATERIAL AND METHODS

General schema. Human colon cancer cell lines were screened for expression of VEGF, and high and low expressors of VEGF were identified. Before we performed antisense transfections, it was necessary to determine which of the four transcripts of VEGF was most abundantly expressed. Reverse transcriptase–polymerase chain reaction (RT-PCR) was used to determine the relative abundance of the splice variants of VEGF. The complementary DNA (cDNA) for the most abundant splice variant was then subcloned into a eukaryotic expression vector; its orientation was determined by means of DNA sequencing and restriction enzyme analysis. The antisense construct was then transfected into the cell line that expresses high amounts of VEGF, and the sense construct was transfected into the cell line that expresses low amounts of VEGF. VEGF secretion into the medium of transfected cells was then quantitated by Western blot analysis to determine whether antisense transfection decreased VEGF protein secretion or sense transfection increased protein secretion. Conditioned medium from these cell lines was then added to endothelial cells in culture so we could study the paracrine effects of colon cancer VEGF expression on endothelial cell proliferation.

Human colon cancer cell lines. The following human colon cancer cell lines were obtained from the American Type Culture Collection (Rockville, Md.) and grown under the supplier's recommended conditions: SW480, SW620, SNUC2B, HT-29, T-84, NCI-H747, NCI-H508, and LoVo. KM12C, KM12SM, KM12L4, and KM20 were a gift from I. J. Fidler, DVM, PhD (M. D. Anderson Cancer Center, Houston, Texas) and were grown in 10% fetal bovine serum (Gibco, Grand Island, N.Y.) minimum essential medium (MEM) at 37° C in 5% CO_2 supplemented with glutamine, sodium pyruvate, vitamins, and nonessential amino acids.[13] Cells were free of *Mycoplasma*, reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Bethesda, Md.).

mRNA extractions and Northern blot analysis. Polyadenylated mRNA was extracted from 10^7 to 10^8 tumor cells growing in culture by using FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, Calif.). Three micrograms mRNA were fractionated on 1% denaturing formaldehyde/agarose gels, transferred to Hybond nylon membrane (Amersham Corp., Arlington Heights, Ill.) by capillary elution, and ultraviolet cross-linked with 120,000 microjoules/cm^2 by using an ultraviolet Stratalinker 1800 (Stratagene, La Jolla, Calif.). After prehybridization the membranes were probed for VEGF and reduced glyceraldehyde-phosphate dehydrogenase (GAPDH). Each cDNA probe was purified by agarose gel electrophoresis, recovered by using QIAEX Gel Extraction kit (QIAGEN, Inc., Chatsworth, Calif.), and radiolabeled by random primer technique with a commercially available kit (Amersham Corp.) with deoxycytidine triphosphate α-^{32}P (Amersham Corp.). Nylon filters were washed at 65° C with 30 mmol/L NaCl, 3 mmol/L sodium citrate (pH 7.2), and 0.1% sodium dodecyl sulfate (w/v). Autoradiography was then performed.

A 1.28 kilobase GAPDH probe, a gift from Robert Radinsky, PhD (M. D. Anderson Cancer Center), was used as an internal control. The VEGF probe, a 204 base pair fragment of human VEGF (VPF) cDNA, was a gift from Brygida Berse, PhD (Harvard Medical School, Boston, Mass.).[7]

RT-PCR to study alternate splicing of VEGF. One microgram total RNA was used for cDNA synthesis with avian myeloblastosis virus reverse transcriptase (50 units; Gibco-BRL) in 20 µl final volume. The reaction mixture included 0.5 mol/L Tris-HCl (pH 8.0); 0.5 mol/L KCl; 0.1 mol/L dithiothreitol; 0.05 mol/L $MgCl_2$; and deoxyribonucleotides (2.5 mmol/L, deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate), RNasin (40 units; Boehringer Mannheim, Indianapolis, Ind.), reverse transcriptase (50 units; Gibco-BRL), and 0.5 µg random primers. The cDNA synthesis reaction was for 1 hour at 37° C. A portion of the reac-

tion mixture (3 μl) was subjected to PCR amplification in a reaction mixture (50 μl) that contained 1 μmol/L each of two primers, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each of four deoxynucleotides, and 2.5 units Taq polymerase (Promega, Madison, Wisc.). PCR amplification of VEGF cDNA was performed in triplicate under the following conditions: 30 cycles, 1 minute at 93° C, 1 minute at 65° C, 1 minute at 72° C. The PCR primers (sense 5'-TCC AGG AGT ACC CTG ATG AG-3', antisense 5'-TTC TGT ATC AGT CTT TCC TGG TGA G-3') immediately flank the region of the VEGF open reading frame involved in the alternative splicing of several exons.[14]

Subcloning of VEGF-121 into pcDNA3 and DNA transfection. The full-length cDNA for VEGF-121 was a gift from Judith Abraham, PhD (Scios Nova, Mountain View, Calif.) and was subcloned into the *Bam*HI site of pcDNA3, a eukaryotic expression vector driven by the human cytomegalovirus promoter (Invitrogen). Subcloning into the *Bam*HI restriction site yielded an insert in either the sense or antisense orientation. The orientation and proof of completeness of the insert were determined by means of restriction enzyme analyses and DNA sequencing (Core sequencing facilities, M. D. Anderson Cancer Center; NIH Core Grant CA 16672-20) (data not shown).

The VEGF-121 sense vector, VEGF-121 antisense vector, and vector alone pcDNA3 were transfected by lipofection according to the manufacturer's protocol (Gibco-BRL). Briefly, 5×10^5 SW480 or SW620 cells were seeded into a 35 mm tissue culture dish and incubated in 1 ml OPTI-MEM I medium (Gibco-BRL) with 5 μl LipoFectin and 1 μg plasmid. Sixteen hours later the OPTI-MEM I medium was replaced with 2 ml fresh growth medium. Drug selection was then started 72 hours after transfection by adding 400 μg/ml G418 in MEM-10% fetal calf serum to the culture. The cells were fed every three days with fresh medium. Three weeks later G418-resistant colonies were transferred separately to an individual well of a 48-well plate, colonies were expanded, mRNA was extracted, and protein was extracted from cell lysates. Protein was also extracted from cell supernatants after growth for 3 days in medium containing 1% fetal bovine serum.

Densitometric quantitation. VEGF protein expression was quantitated by means of densitometry of autoradiograms with the Image Quant software program (Molecular Dynamics, Sunnyvale, Calif.) in the linear range of the film.

Western blot hybridizations. Cells were lysed with protein lysis buffer (20 mmol/L Tris HCl [pH 8.0], 137 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L Na_3PO_4, 2 mmol/L ethylenediamine tetraacetic acid 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and protein was quantitated spectrophotometrically. For cell supernatants the protein was concentrated by using a 10,000 molecular weight concentration column (Amicon, Beverly, Mass.). Then 20 μg aliquots of the protein was separated by electrophoresis on a 12.5% polyacrylamide gel. The protein was transferred to Immobilon-P membrane (Millipore Corp., Bedford, Mass.) by electrotransfer. After blocking with 5% milk in PBS-T (0.1% Tween-20 concentration in phosphate-buffered saline solution), the membrane was probed with the primary antibody (1:200 dilution of polyclonal rabbit antihuman VEGF) (Santa Cruz Biochemicals, Santa Cruz, Calif.). The membrane was washed, and the secondary antibody labeled with horseradish peroxidase (rabbit immunoglobulin from donkey 1:2,000 dilution) (Amersham Corp.) was applied. Protein bands were visualized and densitometry was performed with a commercial chemoilluminesence kit (Amersham Corp.).

Cell proliferation assays. The colon cancer cells lines under study were plated at 50% confluency and were grown in serum-free medium for 3 days in T75 flasks. The supernatant was harvested from the cells and filtered through a 22 μm filter to remove debris and then frozen at -20° C. The conditioned medium was then added to human umbilical vein epithelial cells (HUVEC), and the medium was changed every 48 hours. The MTT (1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan) assay was performed as described below.

HUVEC were obtained from the ATCC and grown on plastic on 0.1% gelatin (Sigma Chemical Co., St. Louis, Mo.) in 10% MEM with supplements (see tumor cell conditions) with epidermal growth factor (10 ng/ml) (Sigma Chemical Co.).

Two thousand cells per well were plated in 96-well plates. At 4 hours (t = 0) and every 24 to 48 hours thereafter (up to 7 days) the MTT assay was done. Then 400 μl of a 2.5 mg/ml solution of MTT was added to wells and incubated for 2 hours at 37° C. The supernatant was removed, and the reaction was stopped with dimethyl sulfoxide, 100 μl/well. The plates were placed on a shaker for 1 minute, and the absorbance was determined on a plate reader read at 570 λ. Each assay was repeated four times. Growth curves were generated by Cricket Graph III (Cricket Software, Malvern, Pa.) computer software.

RESULTS

VEGF mRNA expression in human colon cancer cell lines. Northern blot analyses (Fig. 1) revealed relatively high levels of VEGF expression in 9 of 12 cell lines with low expression in SW480 and very low expression in two metastatic cell lines. Relatively high expression was noted in SW620, which was derived from a metastasis from the patient in whom SW480 was derived from the

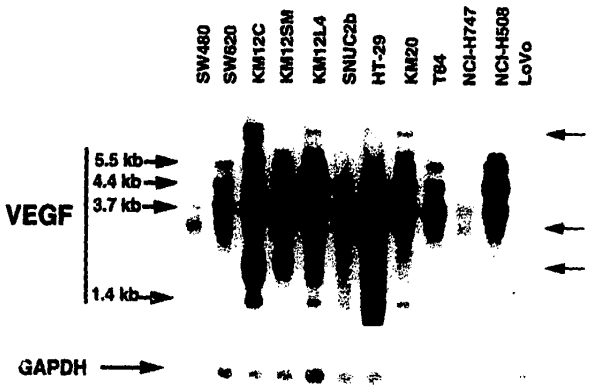

Fig. 1. Northern blot shows VEGF expression in human colon cancer cell lines. Nine of 12 human colon cancer cell lines expressed relatively high levels of VEGF. Autoradiograms for VEGF and GAPDH were exposed for 16 hours and 1 hour, respectively. *Arrows on left* designate major VEGF mRNA transcripts, and *smaller arrows on right* indicate less abundant transcripts that cross-react with VEGF probe.

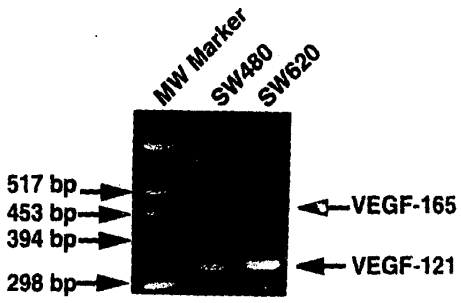

Fig. 2. Alternate splicing of VEGF/RT-PCR analysis. Results of RT-PCR (relative to each cell line) show that VEGF-121 and VEGF-165 are most abundant isoforms expressed in human colon cancer cell lines.

primary tumor. These cell lines were chosen for further study.

RT-PCR. Results of RT-PCR showed that the most abundant splice variants of VEGF in SW480 and SW620 cells were VEGF-121 and VEGF-165, which are the secreted isoforms of VEGF. VEGF-121 was chosen as the isoform to study in transfection experiments (Fig. 2). (It should be noted that this method of PCR amplification does not quantitate between the different cell lines but yields information relative to the most abundant splice variant within each cell line).

VEGF protein expression from human colon cancer cells transfected with VEGF-121 sense and antisense vectors. Cells were grown in serum-free medium for 3 days, and protein was collected from the supernatant. Western blot analyses from cell lysates did not show any differences in VEGF protein levels in the SW480-VEGF sense transfectants when compared with controls or in the SW620-VEGF antisense transfectants when compared with controls. However, when VEGF protein was measured in the supernatant, a threefold increase was noted in the SW480-VEGF sense transfectants, with no change in VEGF protein levels observed in SW480-pcDNA vector transfectants (Fig. 3, *A*). Along the same lines, VEGF protein levels decreased 50% in the SW620-VEGF antisense transfectants, with no change in VEGF protein levels in SW620-pcDNA vector control transfectants (Fig. 3, *B*).

Tumor cell proliferation assay. The following experiment was done to determine that VEGF does not serve as a tumor cell mitogen. The transfected cell lines were grown in 96-well plates in 10% MEM with supplements. The MTT assay was performed as described for endothelial cell proliferation studies; there was no difference in the growth rates of tumor cells secreting various levels of VEGF (data not shown).

Endothelial cell proliferation assay. The addition of conditioned medium from SW480 cells transfected with VEGF-121 resulted in a 1.5-fold increase in endothelial cell proliferation on day 3 and a twofold increase on day 5 compared with endothelial cells grown in con-

ditioned medium from the parental cell line. There was no difference in the proliferation of endothelial cells grown in conditioned medium from the vector-alone transfected SW480 cells and parental SW480 cells (Fig. 4, *A*).

The addition of conditioned medium from SW620 cells transfected with antisense VEGF-121 resulted in a 25% decrease in endothelial cell proliferation on day 2 with a 50% decrease in endothelial cell proliferation on day 6. There was no difference in the proliferation of endothelial cells grown in conditioned medium from the vector-alone transfected SW620 cells and SW620 parental cells (Fig. 4, *B*).

DISCUSSION

Little progress has been made in the treatment of patients with advanced or metastatic colorectal malignancies. The addition of folinic acid to 5-fluorouracil based regimens or alterations in the duration of delivery of 5-fluorouracil has not significantly improved the overall efficacy of systemic chemotherapy.[15] Clearly, new therapeutic regimens must be developed if we are to have any effect on the natural history of this disease.

The tumor microvasculature serves as an attractive target for antineoplastic therapy. Tumor endothelia differ from normal endothelia differ from normal endothelia in that their half-life is on the order of days rather than years.[1] Blood flow and nutrient exchange in tumors are disorderly and inconsistent.[16] In addition, the tumor microvasculature expresses proteins not commonly found in the normal microvasculature.[8, 17] Attacking the tumor microvasculature provides a novel approach to antineoplastic therapy in its simplest form: if tumors do not maintain a conduit for oxygen delivery, then they cannot grow.

Our experiments showed that down-regulation of VEGF in tumor cells can decrease the growth rate of endothelial cells in a paracrine fashion. They raise several issues that warrant discussion. First, transfection of antisense VEGF vectors into human colon cancer cells caused a decrease in VEGF protein in the supernatant of cells. However, we did not note any decrease in VEGF protein in cell lysates. At present the only known functions of VEGF are its effect on endothelia-inducing neovascularization and increased vascular permeability.[11] There is no known autocrine effect; therefore the smaller forms of VEGF (121, 165) are thought to be efficiently secreted into the interstitium. This high degree of protein excretion has been noted in immunohistochemical studies where strong staining for VEGF is noted in the interstitium and on the endothelia (showing binding to its receptor). For secretion from the cell, VEGF does not require transport by binding to another protein, as is the case with basic fibroblast growth factor.[18] Thus we believe that VEGF is synthesized and

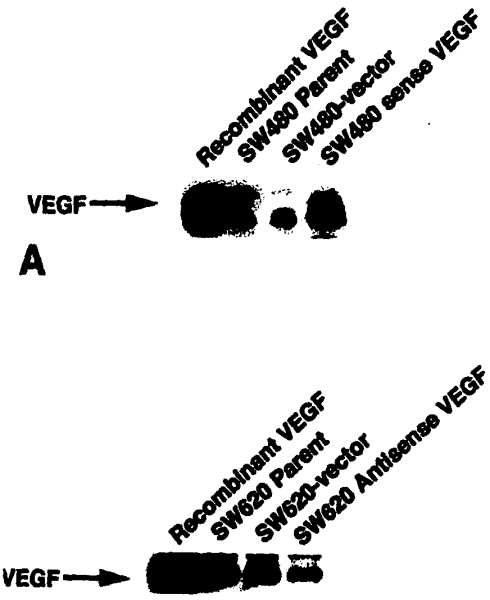

Fig. 3. Western blot of VEGF protein levels in supernatant of cells transfected with sense and antisense VEGF constructs (and vector control [pcDNA3]). **A,** SW480 cells transfected with sense construct for VEGF exhibit threefold increase in VEGF protein in supernatant. **B,** SW620 cells transfected with antisense VEGF construct exhibit 50% decrease in VEGF protein in supernatant.

rapidly secreted into the interstitium; therefore intracellular levels, which are not biologically relevant, may not adequately represent the dynamics of VEGF function.

A second issue raised by these data is that down-regulation of a single angiogenic factor, VEGF, decreased endothelial cell proliferation. This would suggest that in the cell lines studied, VEGF is perhaps the single most important angiogenic factor. Although VEGF may appear to be the predominant angiogenic factor in a pure population of colon cancer cells, tumors are comprised of numerous cell types that may directly or indirectly contribute to the angiogenic phenotype. For example, cytokines and growth factors may alter the expression of VEGF.[19, 20] Other cell types may also contribute to the angiogenic phenotype.[21] By means of immunohistochemical analysis of human colon cancer specimens, we

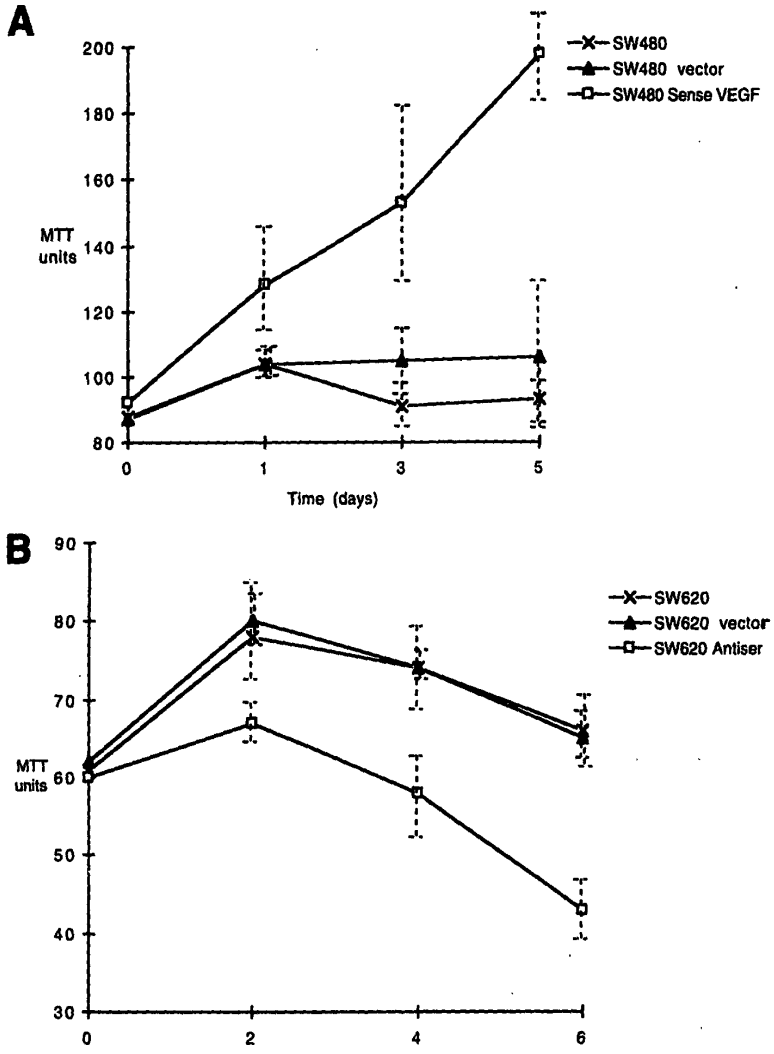

Fig. 4. Proliferation of endothelial cells grown in conditioned medium from experimental cell lines with alteration of VEGF expression. **A,** HUVEC cells cultured in conditioned medium from SW480 cells transfected with sense vector for VEGF-121 exhibit 1.5-fold increase in proliferation by day 1 with twofold increase on day 3 compared with controls. **B,** HUVEC cells cultured in conditioned medium from cells transfected with antisense to VEGF-121 exhibited 25% decrease in proliferation on day 2 with 50% decrease on day 4. (Data points represent mean of each experiment done 4 times; —— represents standard deviation).

have shown that VEGF expression in tumor cells is associated with high vessel counts and metastasis.[12] However, there were some tumors in which the vessel count was high, yet VEGF levels were low. In these tumors we found high expression of another angiogenic factor (platelet-derived endothelial growth fact r) expressed almost exclusively in the infiltrating macrophages and lymphocytes.[22] Thus, although VEGF appears to be an important angiogenic factor in colon cancer, antiangiogenic therapy for this disease must consider the role of other cells present in tumors in the induction of angiogenesis.

Because tumor endothelia have such a short half-life, it is possible that their constant exposure to angiogenic factors is necessary to maintain tumor microcirculation. The importance of VEGF in the maintenance of the tu-

mor vasculature has been well demonstrated. Kim et al.[23] implanted several tumor cell lines into nude mice and treated mice with antibodies to VEGF. Mice treated with the antibody exhibited a decrease in size compared with controls; the magnitude of the decrease in size directly correlated to the level of VEGF in the tumor cell line. Others have also demonstrated a decrease in tumor size with antibodies directed against specific angiogenic factors in various tumors.[24, 25] However, the use of antibodies as therapeutics in human beings is limited by toxicity from the development of human antimouse antibodies.

The process of angiogenesis is driven by the balance of positive and negative regulators of angiogenesis. As with nearly all biologic systems, there is probably redundancy in the regulators of angiogenesis. However, in any one system it is likely that the development of the neovasculature is driven by a predominant factor. Identification of this factor in each tumor type should allow for the rational development of therapy directed against this factor, whether it be the transcription, translation, or neutralization of such a factor, or its biologic effector (i.e., its receptor) on tumor endothelia.

Demonstrating that down-regulation of VEGF can decrease angiogenic activity potentially provides a new target for antineoplastic therapy in patients with colon cancer. It is unlikely that any one factor is entirely responsible for the neovascularization of a human tumor. Our laboratory and others have demonstrated redundancy in biologic processes necessary for tumorigenicity, growth, and metastasis.[26] In addition, it is possible that the host contributes to the angiogenic response by tumors recruiting infiltrating cells that release a myriad of biologic effectors.[27] However, it may be possible to develop an "angiogenic index" for each tumor determining specific and rational targets for antineoplastic therapy.[3] The delivery of agents to inhibit angiogenic factors may be directed toward the synthesis of such factors by using gene delivery systems to inhibit transcription, or, alternatively, the target may be the receptor for a specific angiogenic factor on the tumor microvasculature. Although we used antisense constructs to show the importance of VEGF in the induction of colon cancer angiogenesis, we cannot advocate the development of gene transfer techniques to down-regulate VEGF on the basis of these studies. Gene delivery systems may be developed with antisense constructs or ribozymes, but it is more likely that pharmacologic disruption of the receptor ligand system is a more practical antiangiogenic approach where tumor neovascularization is dependent on VEGF. This is particularly intriguing in tumors dependent on VEGF for angiogenesis because in most organ systems the receptors for VEGF are expressed nearly exclusively on the tumor microvessels. Other solid tumors such as breast cancer and gastric cancer express VEGF; therefore strategies to inhibit tumor angiogenesis through VEGF ligand-receptor inhibition may have application in other tumor systems.

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